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Toxigenesis and biosynthesis of saxitoxin analogues

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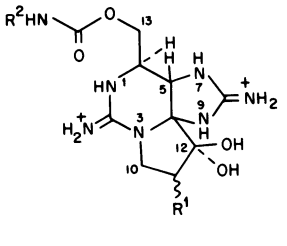
Abstract - The unique tricyclic skeleton of the so-called paralytic shellfish toxins, which include the pharmacologically important saxitoxin and gonyautoxins, was shown to be biosynthesized from arginine and acetate, and the side-chain carbon from methionine by using ^{13}C and ^{13}C-^{15}N doubly-labeled precursors. The historical development of the work and the more basic problem regarding the toxigenicity of the organisms are reviewed.

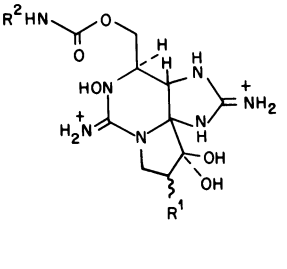
INTRODUCTION

Paralytic shellfish poisoning (PSP) is an acute intoxication caused by the ingestion of toxic bivalves. The problem was first recognized in the temperate waters of North America, Japan, and Europe, but it is now widely found in both northern and southern hemispheres including the tropical areas (ref. 1,2). It is the most damaging problem to the aquaculture of economically important shellfish such as mussels, scallops and oysters. The poisoning involves paralysis of the neuro-muscular system, particularly paralysis of the respiratory system, which is usually the immediate cause of death in cases of severe poisoning.

The chemical studies of PSP toxins started with saxitoxin, which was isolated from the Alaskan butter clam, Saxidomus giganteus in 1957 (ref. 3). After almost twenty years of strenuous studies, the structure of saxitoxin was finally determined by X-ray crystallography (ref. 4,5). Meanwhile, the relationship between the toxification of shellfish and planktonic micro-algae in water was elucidated, and the dinoflagellates, Gonyaulax catenella and G. tamarensis were confirmed as the real toxin-producing organisms (ref. 6).

In 1975, the author's group reported the isolation of several new paralytic shellfish toxins, named gonyautoxins, from the US east coast shellfish samples and from the causative organism, G. tamarensis (ref. 7,8). Subsequently, these toxins and additional new toxins have been isolated from various sources. To date, more than a dozen toxins have been recognized in PSP samples (ref. 2). Structurally, they are divided into two categories: saxitoxin and neosaxitoxin series. In each series, the structural variations are created by the presence and stereochemical difference of the 11-O-sulfate groups. Another interesting





Saxitoxin:	R_1, R_2 = H
Gonyautoxin-II:	R_1 = α-OSO_3^-, R_2 = H
Gonyautoxin-IV:	R_1 = β-OSO_3^-, R_2 = H
Gonyautoxin-V:	R_1 = H, R_2 = SO_3^-
Gonyautoxin-VIII: (= C2 toxin)	R_1 = β-OSO_3^-, R_2 = SO_3^-
Epignyautoxin-VIII: (= C1 toxin)	R_1 = α-OSO_3^-, R_2 = SO_3^-

Neosaxitosin:	R_1, R_2 = H
Gonyautoxin-I:	R_1 = α-OSO_3^-, R_2 = H
Gonyautoxin-IV:	R_1 = β-OSO_3^-, R_2 = H
Gonyautoxin-VI:	R_1 = H, R_2 = SO_3^-
C3 toxin:	R_1 = β-OSO_3^-, R_2 = SO_3^-
C4 toxin:	R_1 = α-OSO_3^-, R_2 = SO_3^-

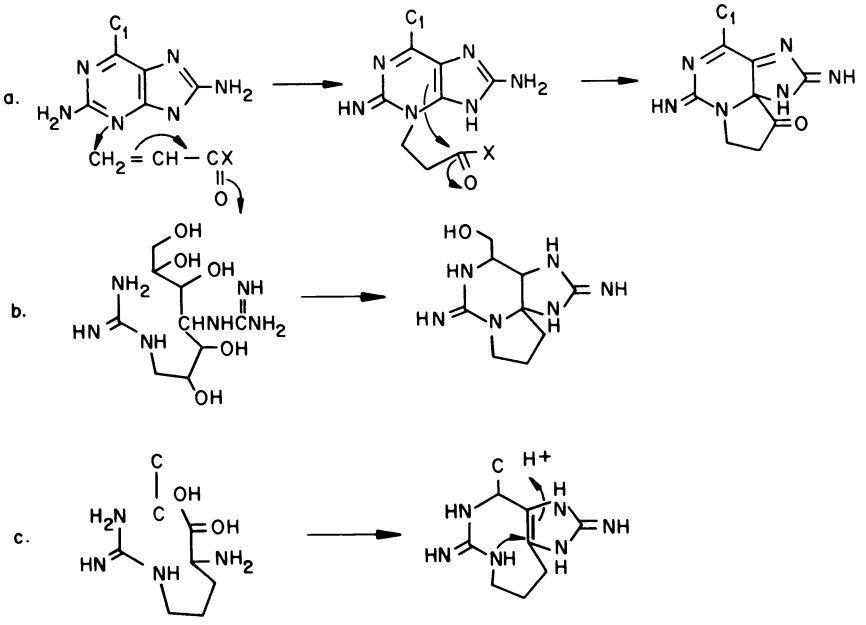
aspect of the structure is the unprecedented occurrence of N-sulfate carbamoyl groups. The N-sulfate toxins can be easily hydrolyzed under very mild acidic conditions to the corresponding free carbamoyl toxins and inorganic sulfate (ref. 9). The structure of neosaxitoxin was proposed to be 1-(N)-hydroxysaxitoxin, (ref. 10) which has recently been confirmed by ^{15}N nmr (ref. 11) and X-ray studies (ref. 12).

Pharmacologically, these natural toxins containing structural variations have proved to be extremely useful for the study of the mechanism of action and the topography of excitable membranes where the toxins react selectively (ref. 3,4,5). Progress has also been made in the study of the causative organisms. We now have a good understanding of the physiology and life-cycle of the organisms. A number of different toxigenic strains and new toxigenic organisms have been discovered. However, despite this flood of information regarding paralytic shellfish poisonings, almost nothing was known until a few years ago about the biosynthesis of the toxin molecules.

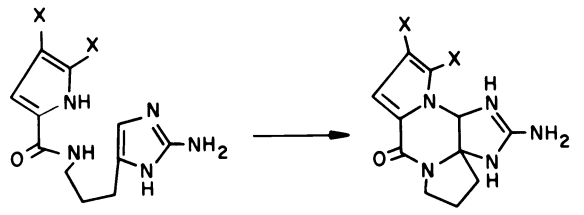
HYPOTHETICAL BIOSYNTHETIC PATHWAYS OF PARALYTIC SHELLFISH TOXINS

There are several speculative pathways referred to publicly or privately by various people - including the present author. The simplest consideration is that the toxin's tricyclic perhydropurine skeleton is derived from the ordinary purine metabolism (ref. 6). In such a scheme, the three-carbon unit which constitutes the third ring system might be formed by Michael addition of acrylate or its equivalent derived from dimethylpropiothetin which is known to be abundant in algal cells (Scheme 1a). Another often suggested idea is to consider the toxin as a derivative of a C_7 sugar. Indeed, a number of aminosugars are

Scheme 1



Scheme 2



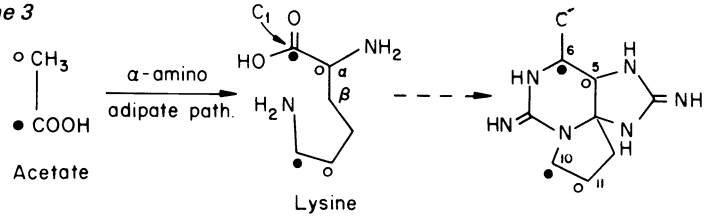
Oroidins: X = H or Br Phakellin

known to carry guanidine or carbamoyl moieties, and simple cyclizations could lead to the toxin skeleton (Scheme 1b). The third scheme, which was the author's preference, is to consider arginine as the major building block, which, with the addition of a C_2 unit or two C_1 units, forms the skeleton (ref. 14). The sequence is analogous to the speculated biosynthesis of phakellin, a sponge metabolite (Scheme 2) (ref. 19).

ORIGINS OF THE RING ATOMS

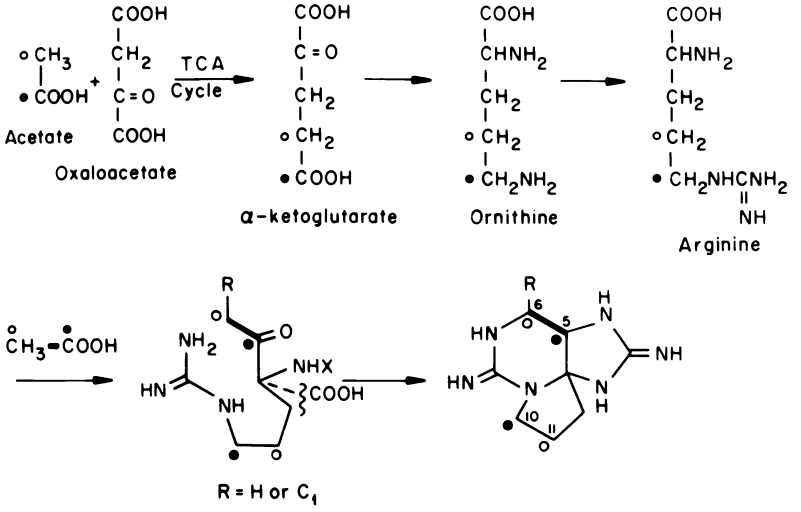
Many feeding experiments were attempted using ^{14}C- and ^{13}C-labeled precursors under various conditions. In most cases, little incorporation or random incorporation of the isotopes was observed. The organism used, G. tamarensis is a nonheterotrophic organism, which generally rejects exogenous organic substances. Also, it was expected that the biosynthesis is highly compartmentallized, and absorbed organic compounds reach the particular synthetic site only after being metabolized to a certain degree. Of a few successful experiments, the feeding of [2-^{13}C]glycine was found to enrich both C-11 and C-12 of gonyautoxin under a certain condition (ref. 14). This apparently unusual enrichment of two adjacent carbons by the single-labeled precursor was explained by the sequence: glycine → glyoxalate → (TCA cycle) → α-ketoglutarate → arginine, which seemed to support the arginine pathway. The feeding of [guanido-^{14}C]arginine resulted in the labeling of the side-chain carbamoyl group (30%) and the ring system (60%), which was confirmed by a degradation study (ref. 14,16). However, attempts to incorporate [1-^{13}C]arginine and [1-^{13}C]ornithine into the toxin molecule failed, which made us try smaller molecules, which might penetrate more easily to the biosynthetic sites. [1,2-^{13}C]Acetate was fed to Gonyaulax cultures maintained under various conditions. The uptake of the acetate was again very limited, but we observed what seemed like the enrichment of C-5 and C-6 of gonyautoxins. The experiment was repeated using Aphanizomenon flos-aquae, a blue-green freshwater alga, which was reported to produce neosaxitoxin and saxitoxin (ref. 20). In this case, the ^{13}C nmr spectrum of the isolated neosaxitoxin clearly showed incorporation of an acetate unit at C_5-C_6 and a lesser amount of another unit at C_{10}-C_{11}. This totally unexpected incorporation pattern was initially explained by the lysine precursor pathway, in which lysine has to be synthesized via the α-aminoadipate pathway, and aminated on the β-carbon (Scheme 3). In fact, the β-amination of lysine is well known, and a very similar imidazoline ring formation on the α- and β-carbons of arginine was demonstrated in the biosynthesis of streptothricin F (ref. 21).

Scheme 3



However, when [2-^{13}C]acetate was fed to the organism in an attempt to confirm the orientation of the incorporated acetate units, signal enhancements were observed for C-6 and C-11, but not for C-5. This raised a doubt about the correct assignment of neosaxitoxin, which was made during its structural determination, by the heteronuclear decoupling of the methine protons which have rather close chemical shifts (ref. 10). In order to establish the assignment unequivocally, the carbon nmr spectrum of randomly enriched neosaxitoxin was examined for the carbon-carbon coupling patterns. This conectivity study confirmed the correctness of the original assignment. Thus, the lysine scheme had to be abandoned and a new pathway was proposed to accommodate the old and new findings (ref. 22) (Scheme 4). In the new scheme, acetate or its derivative undergoes Claisen type condensation on the α-carbon of arginine with a loss of the carboxyl group. The resulting amino-ketone could

Scheme 4

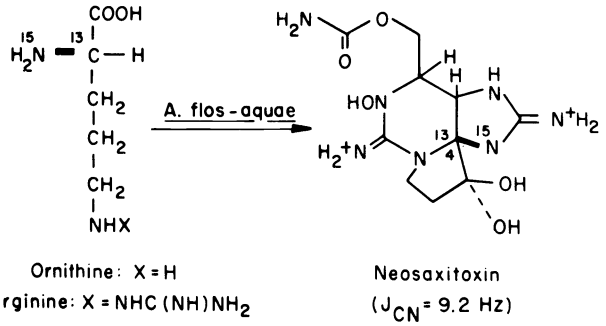


then form the imidazole ring after conversion to a guanido derivative. This key step was proved by the feeding of [2-^{13}C,2-^{15}N]ornithine, which was prepared by the route described by Martinkus et al (ref. 23). Neosaxitoxin isolated from the feeding experiment showed a substantially enriched signal for C-4, which appeared as a doublet by a spin-spin coupling with ^{15}N. The observed J value of 9.2 Hz was in good agreement with the values obtained in an earlier nmr study of ^{15}N-enriched gonyautoxin and neosaxitoxin (ref. 11). The result clearly indicates that the proposed Claisen condensation occurs in the presence of an α-amino group. A well-known analogy is the biosynthesis of δ-aminolevulinate from succinyl CoA and glycine. More recently, the antibiotics arphamenines were reported to be formed by a similar mechanism (ref. 24).

THE ORIGIN OF THE SIDE CHAIN CARBON, C-13

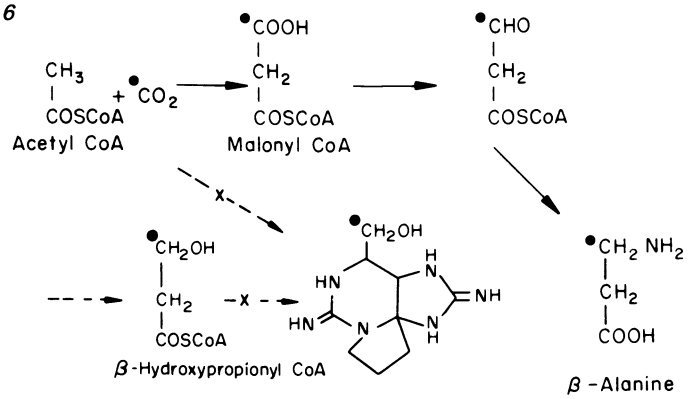
With the origin of all the ring atoms established, the major remaining question was the source of the carbamoyl attached hydroxylmethylene. In an earlier experiment, [^{13}C]formate fed to A. flos-aquae failed to enrich any specific carbon in the toxin molecule. Since formate usually becomes a universal source of C_1 units, we first ruled out such common C_1 donors as methyl and methylene tetrahydrofolate (THF), or S-adenosylmethionine (SAM), and speculated that C-13 could be introduced from CO_2 via the sequence malonyl CoA → semialdehyde → β-hydroxypropionyl CoA (Scheme 5, ref. 25). However, repeated attempts to introduce a specific labeling at C-13 of the toxin by the pulse feeding of $^{13}CO_2$ were unsuccessful. On the other hand, β-alanine, co-existing in the algal cells, was found substantially enriched, indicating that the pulse feeding was actually working, since β-alanine is also biosynthesized from malonsemialdehyde. Similarly, the feeding of [1-^{13}C]-3-hydroxypropionate did not result in a specific incorporation.

Scheme 5

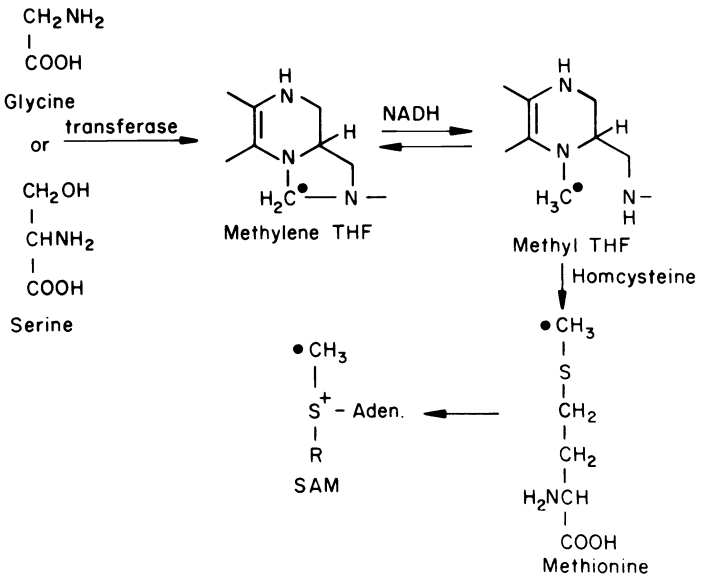


Meanwhile, it was fortuitously discovered that the feeding of [1,2-^{13}C]glyine to A. flos-aquae enriched C-13 of neosaxitoxin quite effectively. The only plausible explanation for such exclusive enrichment of a single carbon by a doubly-labeled precursor is the transfer of the C-2 carbon of glycine to THF by transferase to form methylene THF. To prove this assumption further, we tried the feeding of [3-^{13}C]serine, which is an excellent C_1 donor to THF. The isolated neosaxitoxin was indeed remarkably enriched at C-13. Since methylene THF can be reduced to methyl THF, which is a methyl donor for methionine, that is, for SAM, any of these three compounds can be the direct alkylating agent for the introduction of C-13. When [methyl-^{13}C]methionine was fed to the culture we observed the best incorporation of ^{13}C at C-13 of neosaxitoxin. With this evidence and the fact that the reverse methylation of THF with SAM is uncommon, it is safe to say that SAM is the immediate alkylating agent in the biosynthesis of saxitoxin analogues (Scheme 7).

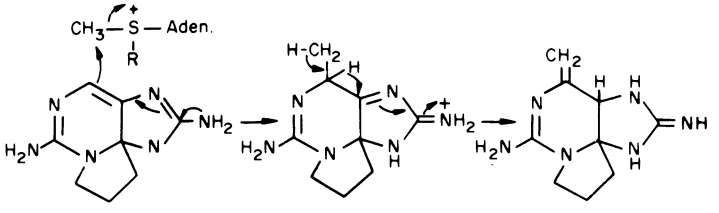
Scheme 6



Scheme 7



The work summarized above proved the biosynthetic origins of all atoms in the saxitoxin skeleton except for the timing and exact mechanism of the cyclization and methylation reactions. However, the most fundamental and intriguing question underlying the whole issue of the biogenesis of these conspicuous toxins - how the organisms come to produce or possess the toxins - remains to be solved. This structurally characteristic group of toxins is now known to be produced by several taxonomically distant organisms: dinoflagellates, fresh-water blue-green alga (cyanobacterium), and even by macro red algae (ref. 26). All of them are normally nontoxigenic or at least known to have the nontoxigenic strains. Thus the cause of the toxigenic properties in the organisms remains unanswered. The presence of endosymbiotic bacteria, or the existence of a trigger mechanism such as seen with the production of phytoallexins are often mentioned. Our extensive study of about 40 strains of



Gonyaulax from various locations seems to suggest that the variance in toxin productivity is not environmental, and also excludes the possibility of the involvement of symbiotic micro-organisms. In the study, the strains were cultured under a strictly controlled condition over years and assayed for their toxigenicity and toxin profile in multiplicates (ref. 27). The results show that the toxigenicity is rather inherent to the strains, and very possibly due to plasmids or some other minor genetic changes. Research efforts are now being focused on the identification of the particular genetic factor common to all these apparently unrelated organisms.

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REFERENCES

1. Y. Shimizu, Marine Natural Products, Chemical and Biological Perspectives, Vol. I, P.J. Scheuer, ed., p. 1, Academic Press, New York (1978).
2. Y. Shimizu, Fortschritte der Chemie Organischer Naturstoffe, Vol. 45, W. Herz, H. Grisebach, C.W. Kirby, eds., p. 235, Springer-Verlag, Wien (1984).
3. E.J. Schantz, J.D. Mold, D.W. Stanger, J. Shavel, F.J. Riel, J.P. Bowden, J.M. Lynch, R.S. Wyler, B. Riegel and H. Sommer, J. Am. Chem. Soc., 79, 5230 (1957).
4. E.J. Schantz, V.E. Ghazarossian, H.K. Schnoes, F.M. Strong, J.P. Springer, J.O. Pezzanite and J. Clardy, J. Am. Chem. Soc.., 97, 1238 (1975).
5. J. Bornder, W.E. Thiessen, H.A. Bates and H. Rapoport. J. Am. Chem. Soc., 97, 6008 (1975).
6. E.J. Schantz, Microbial Toxins, Vol. II, S. Kadis, A. Ciegler, S.J. Agil, eds., Academic Press, New York (1972) and references therein.
7. Y. Shimizu, M. Alam, Y. Oshima and W E Fallon, Biochem. Biophys. Res. Comm., 66, 731 (1975).
8. Y. Oshima, L.J. Buckley, M. Alam and Y. Shimizu, Comp. Biochem. Physiol., 57C, 31 (1977).
9. M. Kobayashi and Y. Shimizu, J. Chem. Soc. Chem. Commun., 827 (1981).
10. Y. Shimizu, C.-P. Hsu, W.E. Fallon, Y. Oshima, I. Miura and K. Nakanishi, J. Am. Chem. Soc., 100, 6791 (1978).
11. A. Hori and Y. Shimizu, J. Chem. Soc. Chem. Commun., 790 (1983).
12. S. Hall, S.D. Darling, G.L. Boyer, P.B. Reichardt and H.-W. Liu, Tetrahedron Lett., 25, 3537 (1985).
13. C.Y. Kao and S.E. Walker, J. Physiol., 323, 619 (1982).
14. Y. Shimizu, Pure Appl. Chem., 54, 1973 (1982).
15. G. Strichartz, Biophys. J., 33, 209 (1981).
16. Y. Shimizu, M. Kobayashi, A. Genenah and N. Ichihara, Seafood Toxins, ACS Symposium Series, 262, E.P. Ragelis, ed., p. 151, Am. Chem. Soc., Washington, DC (1984).
17. L. Chevolot, Marine Natural Products, Chemical and Biological Perspectives, Vol. IV, P.J. Scheuer, ed., p. 53, Academic Press, New York (1981).
18. G. Sharma and B. Magdoff-Fairchild, J. Org. Chem., 42, 4118 (1977).
19. L.H. Foley and G. Büchi, J. Am. Chem. Soc., 104, 1776 (1982).
20. M. Ikawa, K. Wegener, T.L. Foxall and J.J. Sasner, Jr., Toxicon, 20, 747 (1982).
21. S.J. Gould, K.J. Martinkus and C.-H. Tann, J. Am. Chem. Soc., 103, 2871 (1981).
22. Y. Shimizu, M. Norte, A. Hori, A. Genenah and M. Kobayashi, J. Am. Chem. Soc., 106, 6433 (1984).
23. K.J. Martinkus, C.-H. Tann and S.J. Gould, Tetrahedron, 39, 3493 (1983).
24. S. Ohuchi, A. Okuyama, H. Naganawa, T. Aoyagi and H. Umezawa, J. Antibio., 37, 518 (1984).
25. Y. Shimizu, S. Gupta and M. Norte, J. Am. Chem. Soc., submitted.
26. Y. Kotaki, M. Tajiri, Y. Oshima and T. Yasumoto, Bull. Japan. Soc. Sci. Fish, 49, 283 (1983).
27. L. Maranda, D.M. Anderson and Y. Shimizu, Estuarine, Coastal and Shelf Science, in press.